EPA/OPP MICROBIOLOGY LABORATORY
ESC, Ft. Meade, MD

Standard Operating Procedure
for
Quantitative Suspension Test Method for Determining Tuberculocidal Efficacy of Disinfectants Against *Mycobacterium bovis* (BCG)

SOP Number: MB-16-00

Date Revised: 01-18-06

Initiated By: _____ Date: ___/___/___

Print Name:_____

Technical Review: _____ Date: ___/___/___

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Approved By: _____ Date: ___/___/___

Print Name:_____
Branch Chief

Effective Date: ___/___/___

Controlled Copy No.: _____

Withdrawn By: _____ Date: ___/___/___

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1.0 SCOPE AND APPLICATION:

1.1 This SOP describes the methodology used to determine the efficacy of disinfectants against *Mycobacterium bovis* (BCG) in suspension. This SOP is based on the following guidance documents:

A. New Quantitative Tuberculocidal Procedure- Attachment C of USEPA Data Call-in Notice for Tuberculocidal Effectiveness Data for all Antimicrobial Pesticides with Tuberculocidal Claims, dated June 13, 1986.

B. A More Accurate Method for Measurement of Tuberculocidal Activity of Disinfectants (Ascenzi, J.M., et. al., *Applied Environmental Microbiology*,Vol. 53, No. 9, 1987, pp.2189-2192).

2.0 DEFINITIONS:

2.1 MPB = Modified Proskauer Beck Medium

2.2 MPB/Tween = Modified Proskauer Beck Medium with 0.1% (v/v) Tween 80

2.3 M7H11 = Middlebrook 7H11 Medium

3.0 HEALTH AND SAFETY:

3.1 All manipulations of the test organism are required to be performed in accordance with biosafety practices stipulated in the SOP MB-01, Lab Biosafety. All *M. bovis* (BCG) manipulations are performed in a biosafety level 3 isolation laboratory (i.e., room B202 or room B207).

3.2 Disinfectants may contain a number of different active ingredients, such as heavy metals, aldehydes, peroxides, and phenol. Latex gloves and other personal protective clothing or devices must be worn during the handling of these items for purposes of activation or dilution, or efficacy testing. A chemical fume hood or other containment equipment should be used when performing tasks with concentrated products.

4.0 CAUTIONS:

4.1 To ensure the stability of the test disinfectant solution, perform testing within 3 hours of preparation.

4.2 Strict adherence to the protocol is necessary for valid test results.

4.3 Use appropriate aseptic techniques for all test procedures involving the manipulation

of test organisms and associated test components.

5.0 INTERFERENCES:

5.1 Filters with colonies greater than 30-50 CFU's can be difficult to count. Filters should be checked regularly and those with ≥30 CFU's should be counted every other day once growth is observed by indicating colonies with a marker on the lid of the Petri plate (lids should be immobilized with Petri film). At the end of the incubation period, total counts from all days observed should be recorded on the appropriate form.

6.0 PERSONNEL QUALIFICATIONS:

6.1 Personnel are required to be knowledgeable of the procedures in this SOP. Documentation of training and familiarization with this SOP can be found in the training file for each employee.

7.0 SPECIAL APPARATUS AND MATERIALS:

7.1 Filter Units: Nalgene Sterile Analytical Filter Units (0.45 µm pore size) cat. no. 130-4045 or equivalent

7.2 15 mL glass tissue grinders with glass pestles (Wheaton and/or Kontes)

7.3 Spectrophotometer (Beckman DU Series 500 or equivalent)

7.4 Colony Counter

8.0 INSTRUMENT OR METHOD CALIBRATION:

8.1 Linearity and wavelength verification of the spectrophotometer should be performed according to instructions stipulated in SOP EQ-04, Spectrophotometers.

9.0 SAMPLE HANDLING AND STORAGE:

9.1 Disinfectants are stored according to manufacturers' recommendations or at room temperature if the product label or testing parameters do not specify a storage temperature. Those disinfectants requiring activation or dilution prior to use will only be activated or diluted within three hours of testing or as specified in the product test parameters.

10.0 PROCEDURE AND ANALYSIS:

10.1 Frozen Test Culture Preparation [record all transfers and manipulations on the

Organism Culture Tracking Form for *Mycobacterium bovis* (BCG)]:

TABLE 1. Test Culture Preparation Summary

QSTM Test Culture Preparation			
Step		Description*	Culture Notation§
1.	Monthly Stock M7H9 Slant used to inoculate several tubes of MPB (Sect. 10.1.1)	**Solid→Liquid$_{stationary}$** –the first step in initiating QSTM Test Culture, inoculated tubes incubated slanted stationary until a pellicle forms	-QSTM-01
2.	Pellicle from Step 1 used to inoculate several tubes of MPB/Tween (Sect. 10.1.3)	**Liquid$_{stationary}$→Liquid$_{stationary}$** –the inoculated tubes of MPB/Tween are incubated upright stationary until turbid	-QSTM-02
3.	Stationary MPB/Tween culture used to inoculate flask of MPB/Tween (Sect. 10.1.5)	**Liquid$_{stationary}$→Liquid$_{aerated}$** –5 mL of the stationary MPB/Tween culture is used to inoculate 50 mL flasks of MPB/Tween in duplicate, flasks incubated on orbital shaker (~150 rpm) for 5-7 days	-QSTM-03
4.	Aerated MPB/Tween culture used to inoculate flask of MPB/Tween (Sect. 10.1.7)	**Liquid$_{aerated}$→Liquid$_{aerated}$** –10 or 15 mL of the aerated MPB/Tween culture is used to inoculate 100 or 150 mL flasks of MPB/Tween in duplicate, flasks incubated on orbital shaker (~150 rpm) until OD_{500} is ~0.6	-QSTM-04
5.	Tween 80 added to culture -QSTM-04 (Sect. 10.1.9)	One day prior to harvesting the aerated flask culture from step 4 (-QSTM-04), Tween 80 is added (1 mL per liter of culture)	N/A
6.	Culture Harvest (Sect. 10.1.10)	Cells are harvested when OD_{500} is ~0.6 by homogenization in a tissue grinder	N/A
7.	Frozen Test Culture (Sect. 10.1.13)	Pooled homogenized culture is dispensed into cryovials and frozen at ≤-70ºC	-QSTM-FTC

* all incubations performed at 37±1ºC
§ Culture notations should be added to the "Comments" section of the Organism Culture Tracking Form for *Mycobacterium bovis* (BCG)

10.1.1 Inoculate several 10-20 mL tubes of Modified Proskauer-Beck (MPB) medium with *Mycobacterium bovis* (BCG) from a monthly stock M7H9 slant culture (see SOP MB-02, Test Microbes).

10.1.2 Incubate in a slanted position at 37±2°C until a pellicle forms

(approximately 21-25 days).

NOTE: Alternately, the 21-25 day old weekly MPB transfer cultures that were inoculated from M7H9 stock slants (used for Confirmatory Tuberculocidal Efficacy Testing) may also be used.

10.1.3 Transfer a loopful of pellicle onto the surface of several 10-20 mL tubes of Modified Proskauer-Beck with 0.1% (v/v) Tween 80 (MPB/Tween 80).

10.1.4 Incubate stationary at 37±2°C until cultures are turbid. Cultures will require agitation (by gentle shaking/vortexing) to assess turbidity.

10.1.5 Transfer 5 mL of a stationary culture to 50 mL of MPB/Tween 80 in a 250 mL flask. Perform this step in duplicate (as a backup).

10.1.6 Incubate for 5-7 days at 37±2°C with aeration (on a shaker at slow speed, approximately 150 rpm).

10.1.7 Transfer 10 mL of the aerated culture to 100 mL of MPB/Tween 80 in a 500 mL flask. Perform this step in duplicate (as a backup). Alternately: Transfer 15 mL of the aerated culture to 150 mL of MPB/Tween 80 in a 500 mL flask. Perform this step in duplicate (as a backup).

10.1.8 Incubate for 10-15 days at 37±2°C with aeration (on a shaker at slow speed, approximately 150 rpm) OR until the absorbance at 500 nm is about 0.6 (1-5 $\times$ 10^8 CFU/mL- this is the stock culture target density).

10.1.9 One day prior to harvesting, add Tween 80 to the culture (1 mL per L of culture).

10.1.10 Harvest cells when absorbance at 500 nm is approximately 0.6.

10.1.11 Homogenize 10-20 mL aliquots in a tissue grinder.

10.1.12 Pool homogenized culture.

10.1.13 Dispense 1-2 mL aliquots of the homogenized suspension into cryotubes.

10.1.14 Place in cryostorage at ≤-70°C. The concentration of viable cells in the suspension should be checked by plating dilutions of the stock on

M7H11 agar or M7H9 agar plates both before and after freezing. Check the frozen test culture stock by acid-fast staining.

10.2 Suspension Test Culture Preparation:

10.2.1 To prepare the suspension of *M. bovis* (BCG), remove the necessary number of vials of frozen stock culture and place on ice.

10.2.2 Thaw the frozen vials in a 37ºC water bath. Place the thawed vials back on ice. The vials should be thawed quickly and monitored during the thawing process to insure they are placed on ice immediately upon thawing. A vial of ~1.8 mL of frozen test culture requires ~90-120 seconds to thaw completely. Details of the thawing process should be recorded on the QSTM: Test Culture- *M. bovis* (BCG) & Equipment Sheet.

10.2.3 Add an equal volume of buffered gelatin to the suspension and homogenize with a sterile tissue grinder for 1 minute while keeping the culture at 0-4°C in an ice bath.

10.2.4 Dilute the homogenate with sterile saline plus 0.1% Tween 80 to achieve the target density of approximately $1\text{-}5\times10^7$ CFU/mL.

10.2.5 If organic soil is specified in the test parameters for the product test, measure the culture and add the appropriate volume of soil to the diluted homogenate. Swirl to mix.

10.3 Disinfectant Sample Preparation:

10.3.1 Turn on the recirculating chiller and allow the temperature of the chiller unit and the test tube water bath to equilibrate. The temperature should be 20±1ºC or the temperature at which the product is to be tested as specified in the test parameters. Record the temperatures on the Quantitative Suspension Test Method Information Sheet (see 16.3).

10.3.2 Follow chain of custody guidelines for disinfectant samples as stipulated in SOP COC-01, Sample Login and Tracking.

10.3.3 Ready-to-use products are tested as received; no dilution is required.

10.3.4 Prepare disinfectant samples according to the specified test parameters. Record specific test parameters on the Quantitative Suspension Test Method Information Sheet (see 16.3).

10.3.5 To ensure stability, prepare the disinfectant dilutions within three hours of testing or as specified in the test parameters for the product.

10.3.6 Prepare all dilutions with appropriate glassware.

10.3.7 Prior to opening the container of a liquid product, gently shake the container and thoroughly clean the area around the cap and spout with 70% ethanol. Allow the surface to dry. Remove the cap. Do not touch the inside surface of the cap. If present, carefully remove the seal attached to the top of the spout with sterile instruments (i.e., razor blade, forceps).

10.3.8 Pour an appropriate aliquot of the sample into a sterile beaker. Do not place a pipette or any other instrument inside the product container. Place the cap on the product container and secure tightly. From the beaker, dispense ready-to-use products directly into sterile medication tubes or initiate dilutions for diluted products.

10.3.9 For diluted products, use 1.0 mL or more of sample disinfectant to prepare the dilution to be tested. Use v/v dilutions for liquid products and w/v dilutions for solids. Round to two decimal places toward a stronger product. Record the preparation on the Media Reagent Preparation Sheet (see SOP MB-10, Media and Reagents Used in Efficacy Testing).

10.3.10 Once the dilutions are prepared, dispense 9 mL aliquots of the disinfectant into sterile 20×150 mm tubes.

10.4 Test Procedure:

10.4.1 Suspension Test Procedure (see Attachment 1, Study Design for QSTM Disinfectant Efficacy Evaluation):

10.4.1.1 Allow four (one tube per replicate) 20×150 mm tubes each containing 9.0 mL of prepared germicide to equilibrate for 10 minutes at 20±1ºC in a water bath.

10.4.1.2 In a timed step, add 1.0 mL of the test culture to each tube of germicide, and mix by light vortexing. Four replicates are necessary; thus, this step will be repeated four separate times.

10.4.1.3 Following the specified exposure period (tracked by

use of on official timer), remove a 1.0 mL aliquot of the germicide-organism mixture and transfer directly to a 9.0 mL tube of neutralizer (the 1×10^{0} dilution designated Tube A) and mix thoroughly.

10.4.1.4 Within 5 minutes of the transfer to the neutralizer tube, make two additional ten fold dilutions in saline blanks to achieve 1×10^{-1} and 1×10^{-2} dilutions (designated Tube B and Tube C respectively); mix thoroughly between dilutions.

10.4.1.5 The three dilutions (tubes A, B, and C) are filtered separately. To filter, add 20 mL sterile saline into each membrane filter apparatus. Pipette 1 mL of the 1×10^{0} (Tube A), 1×10^{-1} (Tube B), and 1×10^{-2} (Tube C) dilutions into the saline in each of the three filter unit. Briefly swirl and activate the vacuum until all the liquid passes through the filter.

10.4.1.6 Rinse each filter with 50 mL saline.

10.4.1.7 Aseptically disassemble the filter units and transfer individual filters to the surface of M7H11 agar plates. Incubate at 37±1ºC for 21-28 days (plates should be sealed to prevent dessication).

10.4.2 Enumeration of Inoculum (see Attachment 2, Study Design for QSTM Culture Titer and Controls):

10.4.2.1 Transfer 1.0 mL of the test culture (with soil if specified) to a 9.0 mL saline blank and vortex.

10.4.2.2 Perform serial 10 fold dilutions in saline to achieve dilutions through 1×10^{-7}.

10.4.2.3 Filter dilutions by transferring 20 mL saline to each disposable filter unit (8 total).

10.4.2.4 Add 1.0 mL of each dilution (1×10^{-4} through 1×10^{-7} dilutions in duplicate) into the 20 mL saline in the filter unit.

10.4.2.5 Briefly swirl the filter unit. Attach the filter to a vacuum source and activate vacuum until all liquid

passes through the filter.

10.4.2.6 Rinse each filter with 50 mL saline.

10.4.2.7 Aseptically disassemble the filter units and transfer individual filters to the surface of M7H11 agar plates. Incubate at 37±1ºC for 21-28 days (plates should be sealed to prevent dessication).

10.4.3 This SOP describes the methodology for determining disinfectant efficacy at one specified exposure period. A survival curve can be generated for *M. bovis* (BCG) (percent survivors vs. time) after exposure to a concentration of the particular germicide for various contact times. For multiple exposure periods, Section 10.4.1 can be repeated for each additional exposure period. Include the details for multiple exposure periods in the Study Protocol. For additional information, see Reference 15.1, A More Accurate Method for Measurement of Tuberculocidal Activity of Disinfectants (Ascenzi, J.M., et. al., *Applied Environmental Microbiology*,Vol. 53, No. 9, 1987, pp.2189-2192).

10.5 Quality Control:

10.5.1 Static Control: The Static Control is designed to confirm the neutralization of the test substance (see Attachment 2, Experimental Design for QSTM Culture Titer and Controls).

10.5.1.1 Allow 0.9 mL of use-dilution germicide to come to the specified test temperature in a water bath.

10.5.1.2 9.0 mL of neutralizer is added and mixed by vortexing.

10.5.1.3 After 5 minutes, 0.1 mL of the test culture is added and mixed by vortexing.

10.5.1.4 Prepare ten fold dilutions in saline 1×10^{-1} through 1×10^{-5}.

10.5.1.5 Filter dilutions 1×10^{-2} through 1×10^{-5} in duplicate as indicated in Sections 10.4.2.3 – 10.4.2.7 (this will result in 8 plates total).

10.5.2 Neutralizer Toxicity Control: The Neutralizer Toxicity Control must demonstrate that the neutralizer does not impact the recovery of the

test organism (see Attachment 2, Experimental Design for QSTM Culture Titer and Controls).

10.5.2.1 Add 1.0 mL of the standardized test culture to a tube containing 9.0 mL of saline at room temperature.

10.5.2.2 Remove 1.0 mL (of the saline/test culture mixture) and add to a tube containing 9.0 mL neutralizer and mix.

10.5.2.3 After a 5 minute contact period, prepare ten fold dilutions in saline 1×10^{-1} through 1×10^{-5}.

10.5.2.4 Filter dilutions 1×10^{-2} through 1×10^{-5} in duplicate as indicated in Sections 10.4.2.3 – 10.4.2.7 (this will result in 8 plates total).

10.6 Reading Filters and Recording Results:

10.6.1 Filters may be examined after approximately 10 days and daily after that (see Interferences section 5.1).

10.6.2 Colonies appear initially as small buff colored accretions with irregular borders and should be counted using a dissecting microscope and lateral lighting to aid in visualization. Record colony counts at the end of the incubation period on appropriate test sheets.

10.7 Confirmation Procedures and Identification of *M. bovis* (BCG):

10.8.1 The confirmatory tests used to verify the identity of *M. bovis* (BCG) are acid fast staining and plating on selective media.

10.8.2 A smear for acid fast staining is taken from a representative colony from selected filters with growth on the day that final results are recorded. For each set of filters from the Product Test, Enumeration of Inoculum, Static Control, and Neutralizer Toxicity Control, choose the filter with growth from the highest dilution (i.e., the smallest number of colonies).

10.8.3 Acid fast rods are typical for *M. bovis* (BCG). Record results on the Worksheet for Recording Gram Stain and Acid Fast Reactions.

10.8.4 In addition, the representative growth from the colony that was used for Acid Fast staining is streaked over the surface of a M7H11 agar plate, a selective medium, and incubated for 21-25 days at 37±1ºC.

10.8.5 Following the 21-25 day incubation period, the colony morphology of the organism on M7H11 agar should be evaluated. *M. bovis* (BCG) typically appears as colorless to buff-colored, raised, rough growth on M7H11 agar (see SOP MB-02, Test Microbes).

10.8.6 Record confirmation results on the Test Microbe Confirmation Sheet (see 16.6)

11.0 DATA ANALYSIS/CALCULATIONS: See 16.13, QSTM: Calculations Worksheet.

11.1 The test substance must demonstrate $\geq 1.0\times10^4$ CFU kill of the test organism at the stated contact time (i.e., a ≥ 4 $\log_{10}$ reduction of test organism).

11.2 The Static Control (A) should demonstrate that the neutralizer adequately neutralized the test substance (i.e., ≤ 1 $\log_{10}$ difference between A & B).

11.3 The Neutralizer Toxicity Control (B) must demonstrate that the neutralizer does not impact the recovery of test organism (i.e., ≤ 1 $\log_{10}$ difference between B & C).

11.4 The Organism Titer (C) must be $\geq 1\times10^7$ CFU/mL.

12.0 DATA MANAGEMENT/RECORDS MANAGEMENT:

12.1 Data will be recorded promptly, legibly, and in indelible ink on the appropriate forms. Completed forms are archived in notebooks kept in secured file cabinets in D217. Only authorized personnel have access to the secured files. Archived data is subject to OPP's official retention schedule contained in SOP ADM-03, Records and Archives.

13.0 QUALITY CONTROL:

13.1 For quality control purposes, the required information is documented on the appropriate form(s) (see 16.0).

14.0 NONCONFORMANCE AND CORRECTIVE ACTION:

14.1 Any deviation from the standard protocol and the reason for the deviation will be recorded on the appropriate record sheet (see 16.0); corrective action will be expeditious and will be reported under the Good Laboratory Practice Statement in the final report.

15.0 REFERENCES:

15.1 A More Accurate Method for Measurement of Tuberculocidal Activity of Disinfectants (Ascenzi, J.M., et. al., *Applied Environmental Microbiology*,Vol. 53, No. 9, 1987, pp.2189-2192).

15.2 New Quantitative Tuberculocidal Procedure- Attachment C of USEPA Data Call-in Notice for Tuberculocidal Effectiveness Data for all Antimicrobial Pesticides with Tuberculocidal Claims, dated June 13, 1986.

16.0 FORMS AND DATA SHEETS:

16.1 QSTM: Test Information Sheet

16.2 QSTM: Time Recording Sheet for Quality Controls

16.3 QSTM: Time Recording Sheet for Inoculation and Neutralization Steps

16.4 QSTM: Efficacy Evaluation Results Form

16.5 QSTM: Serial Dilution/Plating Tracking Form for Test Suspension Titer

16.6 QSTM: *M. bovis* (BCG) Suspension Titer Results Form

16.7 QSTM: Serial Dilution/Plating Tracking Form for Static Control

16.8 QSTM: Static Control Results Form

16.9 QSTM: Serial Dilution/Plating Tracking Form for Neutralizer Toxicity Control

16.10 QSTM: Neutralizer Toxicity Control Results Form

16.11 QSTM: Test Microbe Confirmation Sheet

16.12 QSTM: Test Culture- *M. bovis* (BCG) & Equipment Sheet

16.13 QSTM: Calculations Worksheet

Attachment 1 Study Design for QSTM Efficacy Evaluation

Attachment 2 Study Design for QSTM Culture Titer and Controls

QSTM: Test Information Sheet

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		SOP	
Name		Test Date	
Sample No.		Comments/Modifications:	
Lot No.			
Expiration Date			

TEST PARAMETERS/Confirmed by:_____			
H_2O Hardness ($CaCO_3$) ppm	Specified	Titrated(Buret)/Date/Init	HACH/Date/Init
[redacted]		/ /	/ /
Use Dilution	Specified	As Prepared/Date/Init	
[redacted]		/ /	
Organic Soil	Specified	As Prepared/Date/Init	
[redacted]		/ /	
Neutralizer	Specified		
[redacted]			
Temperature (ºC)	Specified	Chiller Display	Test tube Water Bath
[redacted]		Before: After:	Before: After:
Contact Time (min)	Specified	As Tested	
[redacted]			
Other Parameters	Specified		
[redacted]			

TEST MICROBE INFORMATION/Confirmed by:_____	
Test Organism	*Mycobacterium bovis* (BCG)
Org. Control No.	
Avg. CFU/mL	

REAGENT/MEDIA INFORMATION/Confirmed by:_____			
Reagent/Media	Prep. No.	Reagent/Media	Prep. No.

QSTM: Time Recording Sheet for Quality Controls

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____	
Test Date	
Product Reg. No.	
Product Name	
Sample No.	

Quality Controls	1st Addition♦	2nd Addition or End Time♦♦	Filtration End Time**
	Start time*	Start time*	Clock Time / Init.
A. Static Control	/	/	/
B. Neut. Tox. Control	/	/	/
Comments:			
♦ Static Control, first addition is the addition of 9.0 mL neutralizer to germicide.			
Neutralizer Toxicity Control, first addition represents transfer of 1.0 mL of saline/test culture mixture to 9.0 mL of neutralizer			
♦♦ Static Control, second addition is the addition of 0.1 mL of test culture.			
Neutralizer Toxicity Control, there is no second addition, this time represents the end time for the 5 minute incubation of			
saline/test culture and neutralizer.			
* Clock time/timer display ** Time when filtering complete for dilutions			

QSTM: Time Recording Sheet for Inoculation and Neutralization Steps

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____	
Test Date	
Product Reg. No.	
Product Name	
Sample No.	

Replicate # / Initials:	Inoculation of Disinfectant Tube	Neutralization	Filtration End Time**
	Start time*	Start Time*	Time from clock/Initials
Replicate # 1	/00:30	/ :30	/
Replicate # 2	/00:30	/ :30	/
Replicate # 3	/00:30	/ :30	/
Replicate # 4	/00:30	/ :30	/
Comments: * Clock time/timer display			
** Time when filtering complete for dilutions			

QSTM: Efficacy Evaluation Results Form
OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____	
Test Date	
EPA Reg. No.	
Product Name	
Sample No.	

RESULTS Date/Initials:_____

Exposure time/ Dilution-Tube		Colony Forming Unite (CFU)/Membrane				Average CFU/Membrane
		Replicate 1	Replicate 2	Replicate 3	Replicate 4	
	1×10^{0} A					
	1×10^{-1} B					
	1×10^{-2} C					

Comments	

QSTM: Serial Dilution/Plating Tracking Form for Test Suspension Titer

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____	
EPA Reg. No.	
Name	
Sample No. (s)	
Test Date	
Organism	
SOP	

Confirmed by:_____	1	2	3	4	5	6	7
Vol in Dil. Tube prior to Addition	9 mL	9 mL	9 mL	9 mL	9 mL	9 mL	9 mL
Vol. Added to Dil. Tube	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL
Overall Dilution in Dil. Tube	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Volume Filtered	N/A	N/A	N/A	1 mL	1 mL	1 mL	1 mL
Overall Dilution on Filter	N/A	N/A	N/A	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Number of Filters per Dilution	N/A	N/A	N/A	2	2	2	2
Recovery Media for Filters	M7H11						
Comments:							

REAGENT/MEDIA INFORMATION/Confirmed by:_____			
Reagent/Media	Prep. No.	Reagent/Media	Prep. No.

QSTM: *M. bovis* (BCG) Suspension Titer Results Form
OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Name		Test Organism	
Sample No.		Comments:	

RESULTS: Date/Initials:_____

Plating Method			
	CFU per Dilution Plate		CFU per mL of Test Culture
Dilution	Plate 1	Plate 2	
1×10^{-4}			
1×10^{-5}			
1×10^{-6}			
1×10^{-7}			

QSTM: Serial Dilution/Plating Tracking Form for Static Control

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____	
EPA Reg. No.	
Name	
Sample No. (s)	
Test Date	
Organism	
Control	Static Control
SOP	

Confirmed by:_____	1	2	3	4	5	6	7
Vol in Dil. Tube prior to Addition	9 mL	9 mL	9 mL	9 mL	9 mL	N/A	N/A
Vol. Added to Dil. Tube	1 mL	1 mL	1 mL	1 mL	1 mL	N/A	N/A
Dilution in Dil. Tube	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	N/A	N/A
Volume Filtered	N/A	1 mL	1 mL	1 mL	1 mL	N/A	N/A
Overall Dilution on Filter*	N/A	10^{-2}	10^{-3}	10^{-4}	10^{-5}	N/A	N/A
Number of Filters per Dilution	N/A	2	2	2	2	N/A	N/A
Recovery Media for Filters	M7H11						
Comments: * To compare counts to Organism Titer, counts on filters must be multiplied by a Factor of 100.							

REAGENT/MEDIA INFORMATION/Confirmed by:_____			
Reagent/Media	Prep. No.	Reagent/Media	Prep. No.

QSTM: Static Control Results Form
OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
Name		Test Date	
EPA Reg. No.		Test Organism	
Sample No.			

Type of Control	Neutralizer	Germicide	Test Culture	Saline
Static Control	9.0 mL	0.9 mL	0.1 mL	N/A
Static Control: Place germicide in tube and allow to come to temperature in water bath, add				
Neutralizer and mix by vortexing. After 5 minutes, add test culture and mix by vortexing.				
Use 1 mL to make serial dilutions to 1×10^{-5}.				

RESULTS (colony counts)				
Date Read/ Initials:_____				
Type of Control	Dillution			
	1×10^{-2}	1×10^{-3}	1×10^{-4}	1×10^{-5}
Static Control (SC)				
Comments:				

REAGENT/MEDIA INFORMATION/Confirmed by:_____			
Reagent/Media	Prep. No.	Reagent/Media	Prep. No.
M7H11 agar plates			
0.85% Saline Blanks			
0.85% Saline (rinse)			

QSTM: Serial Dilution/Plating Tracking Form for Neutralizer Toxicity Control
OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____	
EPA Reg. No.	
Name	
Sample No. (s)	
Test Date	
Organism	
Control	Neutralizer Toxicity Control
SOP	

Confirmed by:_____	1	2	3	4	5	6	7
Vol in Dil. Tube prior to Addition	9 mL	9 mL	9 mL	9 mL	9 mL	N/A	N/A
Vol. Added to Dil. Tube	1 mL	1 mL	1 mL	1 mL	1 mL	N/A	N/A
Dilution in Dil. Tube	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	N/A	N/A
Volume Filtered	N/A	1 mL	1 mL	1 mL	1 mL	N/A	N/A
Overall Dilution on Filter*	N/A	10^{-2}	10^{-3}	10^{-4}	10^{-5}	N/A	N/A
Number of Filters per Dilution	N/A	2	2	2	2	N/A	N/A
Recovery Media for Filters	M7H11						
Comments: * To compare counts to Organism Titer, counts on filters must be multiplied by a Factor of 100.							

READENT/MEDIA INFORMATION/Confirmed by:_____			
Reagent/Media	Prep. No.	Reagent/Media	Prep. No.
M7H11 agar plates			
0.85% Saline Blanks			
0.85% Saline (rinse)			

QSTM: Neutralizer Toxicity Control Results Form
OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
Name		Test Date	
EPA Reg. No.		Test Organism	
Sample No.			

Type of Control		Neutralizer	Germicide	Test Culture	Saline
Neutralizer Toxicity Control	A*	N/A	N/A	1.0 mL	9.0 mL
	B*	9.0 mL	N/A	1 mL of test culture/saline mixture	
Neutralizer Toxicity Control: *Step A: Test culture is added to tube containing saline at room					
temperature. The tube is mixed by vortexing. Step B: 1.0 mL of the test culture/saline mixture is					
transferred to a tube containing 9.0 mL neutralizer. After about 5 minutes, 1.0 mL is used to make					
Serial dilutions in saline. Use 1 mL to make serial dilutions to 1×10^{-5}.					

RESULTS (colony counts)				
Date Read/ Initials:_____				
Type of Control	Dillution			
Neutralizer Toxicity Control (NTC)	1×10^{-2}	1×10^{-3}	1×10^{-4}	1×10^{-5}
Comments:				

REAGENT/MEDIA INFORMATION/Confirmed by:_____			
Reagent/Media	Prep. No.	Reagent/Media	Prep. No.
M7H11 agar plates			
0.85% Saline Blanks			
0.85% Saline (rinse)			

QSTM: Test Microbe Confirmation Sheet
OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Name		Test Organism	*M. bovis* (BCG)
Sample No.		Comments:	

Source: Tube/Plate ID	Date/ Initials	Stain Results*	Media Information			Results	
			Type	Prep. No.	Inc. Time/ Temp.	Date/ Initials	Colony Characteristics

* Record AFR for Acid Fast Rods

QSTM: Test Culture- *M. bovis* (BCG) & Equipment Sheet
OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____	
Organism/ Control No.	*M. bovis* (BCG)/
Test Date	
Date Frozen	
Date Thawed	
No. Vials Thawed	

Notes:_____

_____

_____

_____

Volume of frozen culture added to tissue grinder:_____

Volume of Buffered Gelatin added to tissue grinder:_____

Buffered Gelatin Prep. No.:_____

Notes:_____

_____

_____

_____

_____

Equipment used in test:

TEST INFORMATION/Confirmed by:_____	
BSC for Testing/Controls	
BSC for Filtration and Plating	
Chiller	
Timer	
Filters:	

QSTM: Calculations Worksheet

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by: _____			
Name		Test Date	
EPA Reg. No.		Test Organism	*Mycobacterium bovis* (BCG)
Sample No.			

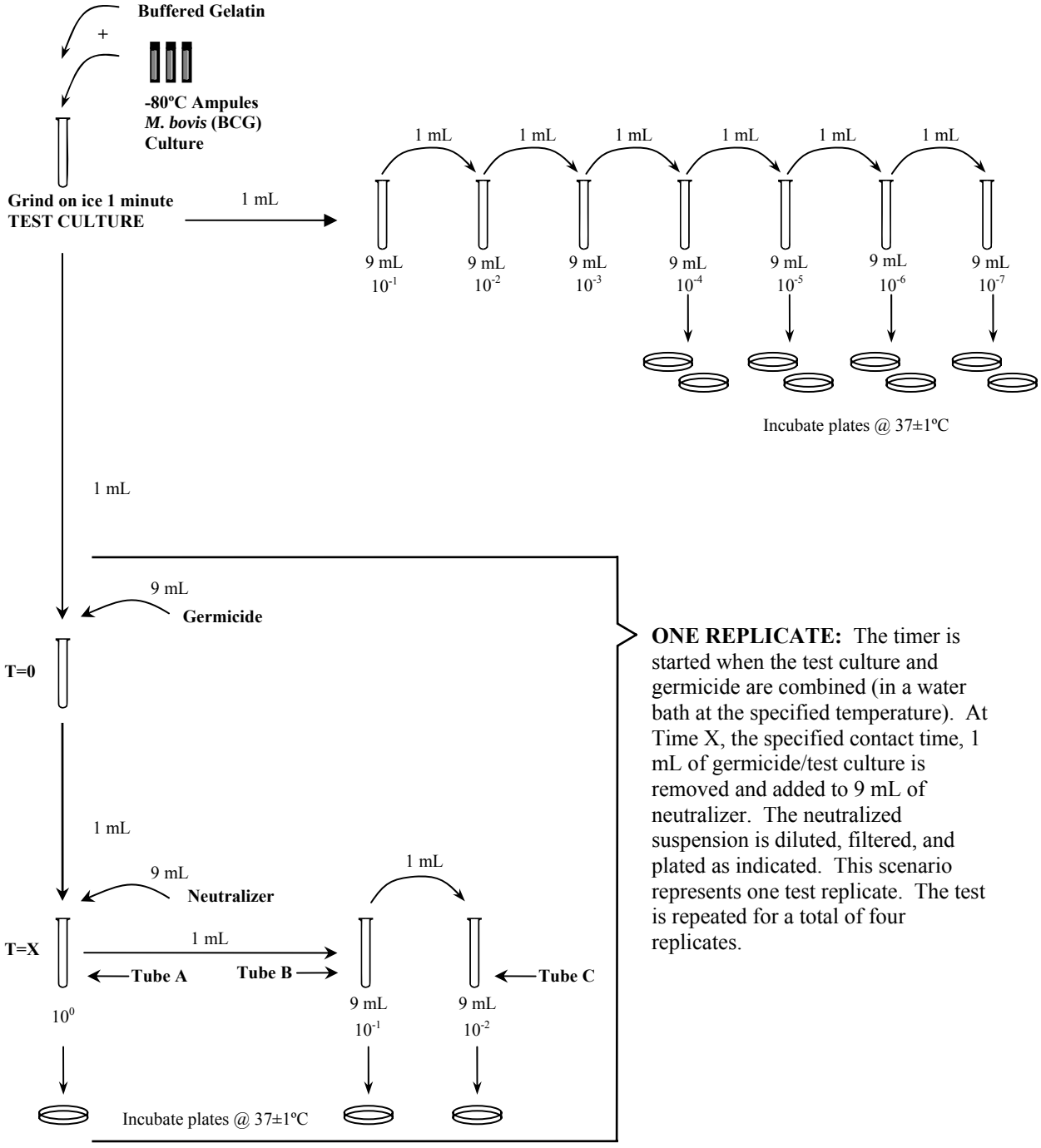
Calculation Summary				
ORGANISM TITER	Dilution			
	1×10^{-4}	1×10^{-5}	1×10^{-6}	1×10^{-7}
CFU/filter	/	/	/	/
Average CFU/filter	a)	b)	c)	d)
CFU/mL = $\frac{(a)+(b)+(c)+(d)}{(1\times10^{-4})+(1\times10^{-5})+(1\times10^{-6})+(1\times10^{-7})}$ =				
Log_{10} CFU/mL				
STATIC CONTROL	Dilution			
	1×10^{-2}	1×10^{-3}	1×10^{-4}	1×10^{-5}
CFU/filter	/	/	/	/
Average CFU/filter	a)	b)	c)	d)
CFU/mL = $\frac{(a)+(b)+(c)+(d)}{(1\times10^{-2})+(1\times10^{-3})+(1\times10^{-4})+(1\times10^{-5})}$ × 100* =				
Log_{10} Static Control				
NEUTRALIZER TOXICITY CONTROL	Dilution			
	1×10^{-2}	1×10^{-3}	1×10^{-4}	1×10^{-5}
CFU/filter	/	/	/	/
Average CFU/filter	a)	b)	c)	d)
CFU/mL = $\frac{(a)+(b)+(c)+(d)}{(1\times10^{-2})+(1\times10^{-3})+(1\times10^{-4})+(1\times10^{-5})}$ × 100* =				
Log_{10} Neutralizer Toxicity Control				

*For direct comparison to the Organism Titer, the control titer must be multiplied by 100.

Disinfectant Log Reduction					
Recovery Results	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Average**
Filter A (1×10^{0})					
Filter B (1×10^{-1})					
Filter C (1×10^{-2})					
Log_{10} Reduction = Log_{10} (Organism Titer)$_{PRE\text{-}TREATMENT}$ − Log_{10} (Organism Titer)$_{POST\text{-}TREATMENT}$					
Log Reduction					

** One CFU recovered on Filter A represents 100 CFU remaining in the disinfectant exposure tube (post-treatment).

Attachment 1
Study Design for QSTM Disinfectant Efficacy Evaluation



ONE REPLICATE: The timer is started when the test culture and germicide are combined (in a water bath at the specified temperature). At Time X, the specified contact time, 1 mL of germicide/test culture is removed and added to 9 mL of neutralizer. The neutralized suspension is diluted, filtered, and plated as indicated. This scenario represents one test replicate. The test is repeated for a total of four replicates.

Attachment 2
Study Design for QSTM Culture Titer and Controls